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Origin plasticity during budding yeast DNA replication *in vitro*

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Abstract

The separation of DNA replication origin licensing and activation in the cell cycle is essential for genome stability across generations in eukaryotic cells. Pre-replicative complexes (pre-RCs) license origins by loading Mcm2-7 complexes in inactive form around DNA. During origin firing in S phase, replisomes assemble around the activated Mcm2-7 DNA helicase. Budding yeast pre-RCs have previously been reconstituted *in vitro* with purified proteins. Here, we show that reconstituted pre-RCs support replication of plasmid DNA in yeast cell extracts in a reaction that exhibits hallmarks of cellular replication initiation. Plasmid replication *in vitro* results in the generation of covalently closed circular daughter molecules, indicating that the system recapitulates the initiation, elongation, and termination stages of DNA replication. Unexpectedly, yeast origin DNA is not strictly required for DNA replication *in vitro*, as heterologous DNA sequences could support replication of plasmid molecules. Our findings support the notion that epigenetic mechanisms are important for determining replication origin sites in budding yeast, highlighting mechanistic principles of replication origin specification that are common among eukaryotes.

Keywords budding yeast; cell cycle; DNA replication; origin; pre-RC
Subject Categories DNA Replication, Repair & Recombination
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Introduction

Eukaryotic DNA replication initiates by a two-step mechanism from many origin sites distributed along the length of each chromosome (Masai *et al*, 2010). In the first step, origins are "licensed" in G1 phase by assembling a pre-replicative complex (pre-RC) that contains the replicative DNA helicase, Mcm2-7, in inactive form. Origin licensing requires the coordinate activities of the six-subunit origin recognition complex (ORC), Cdc6, and Cdt1, which load the Mcm2-7 complex in double-hexameric form around double-stranded DNA (Evrin *et al*, 2009; Remus *et al*, 2009; Gambus *et al*, 2011). Activation of the Mcm2-7 helicase in the second step is restricted to S phase and culminates in the formation of a pair of oppositely oriented replication forks that contain a single Mcm2-7 helicase hexamer complex at the apex of each fork (Boos *et al*, 2012). Mcm2-7 helicase activation depends on the formation of a pre-initiation complex (pre-IC), which coordinates the recruitment of two essential Mcm2-7 helicase co-factors, Cdc45 and GINS, to form the CMG (for Cdc45, Mcm2-7, and GINS) complex. This step is controlled by two protein kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK), and involves essential initiation factors, including the Sld3-Sld7 complex, Dpb11, Sld2, DNA polymerase ε, and Mcm10, whose molecular functions in the process are poorly understood (Araki, 2010; Labib, 2010).

While our understanding of the order by which initiation factors assemble at eukaryotic replication origins has been significantly advanced in recent years, comparatively little is known about how eukaryotic replication origins are specified in *cis* (Gilbert, 2004; Mechali *et al*, 2013). Budding yeast origins were originally identified as "autonomously replicating sequence" (ARS) elements that confer replication competence to episomally maintained plasmids in yeast (Newlon & Theis, 1993). Although ARS elements are relatively short (~150 bp), they do not exhibit extensive sequence conservation apart from a degenerate 11–17 bp ARS consensus sequence (ACS) (Newlon & Theis, 1993; Breier *et al*, 2004; Nieduszynski *et al*, 2006; Xu *et al*, 2006; Eaton *et al*, 2010), which forms part of the ORC binding site (Bell & Stillman, 1992). However, only a small fraction of all matches to the ACS in the budding yeast genome is associated with replication origins, indicating that an ACS is not sufficient to define a budding yeast origin. A variable number of B elements next to the ACS, which are not strictly conserved in sequence, size, or position, are also important for budding yeast origin function, and epigenetic mechanisms are thought to play an additional role in specifying budding yeast origins (Newlon & Theis, 1993; Eaton *et al*, 2010).

In higher eukaryotes, DNA replication initiates from developmentally controlled chromosomal sites that lack conserved sequence elements. Moreover, specific DNA sequences may be altogether dispensable for metazoan origin function, as origin usage is promiscuous during the early embryonic cleavage stages of *Drosophila* and *Xenopus* and during plasmid replication in mammalian cells (Spradling & Orr-Weaver, 1987; Hyrien *et al*, 1995; Schaarschmidt *et al*, 2004), heterologous DNA templates replicate efficiently in

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Xenopus (Harland & Laskey, 1980; Mechali & Kearsey, 1984), and artificial tethering of initiation factors to DNA can be sufficient to specify an origin in *Drosophila* and mammalian cells (Takeda *et al*, 2005; Crevel & Cotterill, 2012). The dependency of origin function on a conserved DNA sequence element, the ACS, in budding yeast has therefore often been contrasted to the heterogeneous origins of higher eukaryotes. The mechanistic basis for this difference is not clear, yet it may be unexpected given the general conservation of basic eukaryotic replication initiation factors.

We and others have previously developed a system to reconstitute pre-RCs with purified budding yeast proteins (Evrin *et al*, 2009; Remus *et al*, 2009). Here, we demonstrate that reconstituted pre-RCs support regulated and complete replication of plasmid DNA in S-phase extracts of budding yeast cells. Using this system, we find that known ARS elements, while being essential for plasmid replication *in vivo*, are not essential *in vitro*. Nonetheless, DNA replication does not initiate indiscriminately with regard to DNA sequence. The data suggest that the replication machinery can access potential origin sites *in vitro* that may otherwise be sequestered *in vivo*. This system, therefore, offers the opportunity to assess the DNA sequence requirements for eukaryotic replication initiation independently of epigenetic regulation, in addition to providing an experimental platform for the biochemical study of initiation factor function.

Results

Reconstituted pre-RCs support DNA replication *in vitro*

To test whether reconstituted pre-RCs are competent for DNA replication, we asked whether they could substitute for G1-phase extracts in an *in vitro* replication approach that recapitulates the origin licensing and activation reactions by transferring immobilized origin-containing plasmid DNA from extracts of yeast cells arrested in G1 phase to extracts of S phase-arrested yeast cells (Heller *et al*, 2011). Pre-RCs were reconstituted on pARS/WTA (Marahrens & Stillman, 1992), a 5.9 kb ARS1-containing plasmid that we refer to as pARS1 here, coupled to paramagnetic beads. Following assembly, pre-RCs were recovered and phosphorylated with purified DDK (Supplementary Fig S1). Pre-RCs were re-isolated and transferred to an S-phase extract prepared from yeast cells containing the *cdc7-4* temperature sensitive allele, which were arrested at non-permissive temperature and which conditionally overproduced the limiting replication initiation factors Sld3, Cdc45, Sld2, and Dpb11. DNA synthesis was assayed by monitoring ^{32}P-dCTP incorporation into purified DNA using alkaline agarose gel electrophoresis and autoradiography. As shown in Fig 1A, this approach led to robust DNA synthesis dependent on both purified ORC and Cdc6. DNA synthesis was inhibited in the absence of DDK, or if the S-phase extract was supplemented with CDK inhibitor Sic1, demonstrating that purified reconstituted pre-RCs can license DNA for cell-free replication in a reaction that bears hallmarks of cellular DNA replication initiation.

ORC, unlike Cdc6 and Cdt1, remains bound at origins after Mcm2-7 loading (Diffley *et al*, 1994; Bowers *et al*, 2004; Evrin *et al*, 2009; Remus *et al*, 2009), and studies in *S. cerevisiae* suggest that ORC is required for both the establishment and maintenance of pre-RCs in G1 phase (Chen *et al*, 2007). On the other hand, ORC can be eluted from licensed chromatin in *Xenopus* without disrupting the replication competence of the chromatin (Hua & Newport, 1998; Rowles *et al*, 1999). Yeast ORC can be eluted from DNA by salt wash without disrupting DNA-bound Mcm2-7 (Donovan *et al*, 1997; Bowers *et al*, 2004; Evrin *et al*, 2009; Remus *et al*, 2009). High-salt conditions, however, induce one-dimensional diffusion of Mcm2-7 double hexamers along the DNA (Evrin *et al*, 2009; Remus *et al*, 2009). We, therefore, asked whether high-salt treatment affects the ability of reconstituted pre-RCs to support DNA replication *in vitro*. As expected, loaded Mcm2-7 proteins are resistant to elution from the circular closed plasmid DNA, while ORC is efficiently eluted at high salt concentration (Fig 1B, lanes 4–6). Importantly, Mcm2-7 complexes largely retained their ability to initiate DNA replication after high-salt treatment (Fig 1B, lanes 1–3). This indicates that ORC does not have to remain in contact with Mcm2-7 for initiation and that Mcm2-7 double hexamers retain their ability to initiate DNA replication after diffusion along DNA.

The incompatibility of G1- and S-phase conditions for origin activation and licensing, respectively, imposes a requirement for the separation of both reaction conditions to initiate DNA replication *in vitro*, which was previously achieved by transfer of immobilized template DNA from G1 phase to the S-phase extract (Heller *et al*, 2011). Because the reconstituted pre-RC assembly reaction lacks inhibitors of origin activation that are present in G1-phase extracts, we hypothesized that simple staging of the pre-RC assembly and origin activation reactions would obviate the need for template transfer. To test this, we assembled pre-RCs on immobilized plasmid DNA, then added DDK directly to the pre-RC assembly reaction, followed by the addition of S-phase extract (Fig 1C). Western blot analysis of the DNA-bound fraction was used to monitor pre-IC assembly. Recruitment of Sld3, Cdc45, Sld2, and Dpb11 was dependent on reconstituted pre-RCs, but was unaffected by the replicative DNA polymerase inhibitor aphidicolin. Recruitment of Sld3 and Cdc45 to the pre-RCs was dependent on DDK, but independent of CDK, consistent with reports that DDK promotes Sld3 and Cdc45 binding to early origins in G1 phase, when CDK activity is low (Aparicio *et al*, 1999; Kamimura *et al*, 2001; Kanemaki & Labib, 2006; Heller *et al*, 2011; Tanaka *et al*, 2011). Recruitment of Sld2 and Dpb11, in contrast, was dependent on both DDK and CDK, consistent with CDK promoting the formation of a ternary complex between Sld2, Sld3, and Dpb11 during initiation (Tanaka *et al*, 2007; Zegerman & Diffley, 2007; Muramatsu *et al*, 2010). These data suggest that reconstituted pre-RCs support the ordered assembly of pre-ICs under our modified conditions. Consistent with previous studies (Heller *et al*, 2011; Watase *et al*, 2012), we observed that efficient recruitment of Mcm10 to the pre-RC was dependent on both DDK and CDK, but independent of DNA synthesis (Fig 1C, lanes 6–10). Conversely, immunodepletion of Mcm10 from the S-phase extract, while greatly reducing DNA synthesis activity, did not eliminate the recruitment of Cdc45, Sld3, or Dpb11 (Supplementary Fig S2A–C), supporting earlier observations that Mcm10 acts after pre-IC and CMG assembly (Heller *et al*, 2011; van Deursen *et al*, 2012; Watase *et al*, 2012). Finally, we tested the replication competence of complexes assembled using the modified approach of Fig 1C. Again, robust DNA synthesis was observed dependent on reconstituted pre-RCs, DDK, and CDK, indicating that our modified protocol recapitulates regulated DNA replication (Fig 1D).

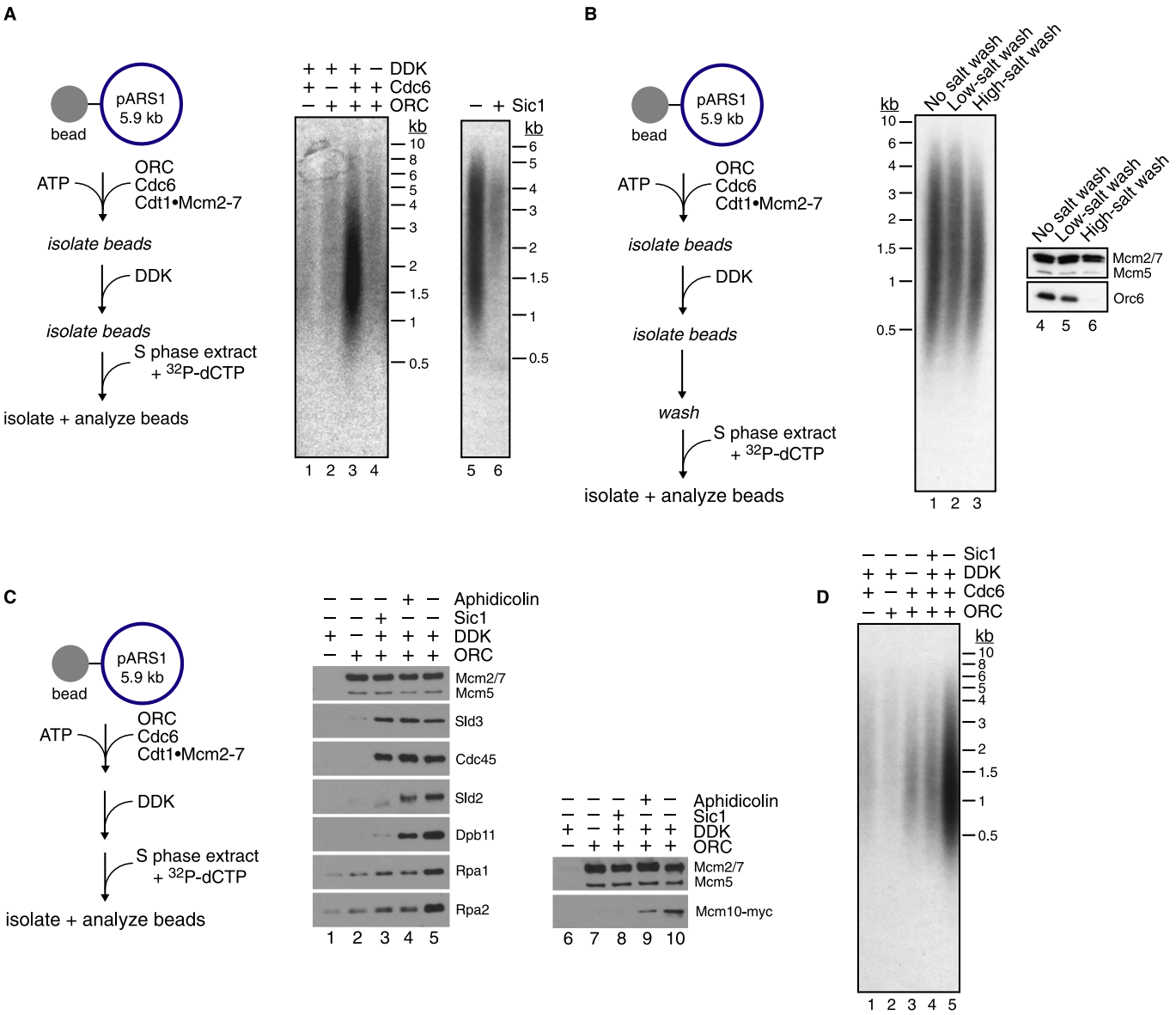

Figure 1. Reconstituted pre-RCs support DNA replication *in vitro*.

A Left: Reaction scheme; blue circle indicates plasmid, gray sphere indicates magnetic bead. Reaction products were analyzed in lanes 1–6 by autoradiography after alkaline agarose gel electrophoresis.

B Left: Reaction scheme. Lanes 1–3: Autoradiogram of replication products fractionated by alkaline agarose gel electrophoresis. Lanes 4–6: Western blot analysis of proteins associated with DNA after wash step. Pre-RCs were treated after Dbf4-dependent kinase (DDK) phosphorylation, as indicated on top, prior to transfer into S-phase extract. Low-salt wash: 0.3 M K-glutamate buffer; high-salt wash: 0.5 M NaCl buffer.

C Left: Reaction scheme. Lanes 1–10: Western blot analysis of indicated proteins associating with DNA beads after 40-min incubation in S-phase extract. Pre-ICs were assembled in S-phase extract prepared from YDR89 (lanes 1–5) or YSD8 (lanes 6–10) cells.

D Autoradiogram of replication products obtained following reaction scheme as in (C). Purified DNA was isolated after the reaction and analyzed by alkaline agarose gel electrophoresis.

Complete replication of plasmid DNA *in vitro*

Because our modified approach obviates the need to transfer template DNA from G1- to S-phase extracts, we repeated the reaction scheme of Fig 1C with pARS1 not bound to beads (Fig 2A). DNA replication products began to appear after a lag phase of 10–20 min, reaching a plateau of DNA synthesis after about 60 min (Fig 2B). Analysis of the replication products by native agarose gel electrophoresis (Fig 2B, lanes 1–5) identified five main replication products: (i) negatively supercoiled plasmid, (ii) nicked plasmid, (iii) linear plasmid, (iv) a distribution of high-molecular weight species (HMW), and (v) products that did not enter the

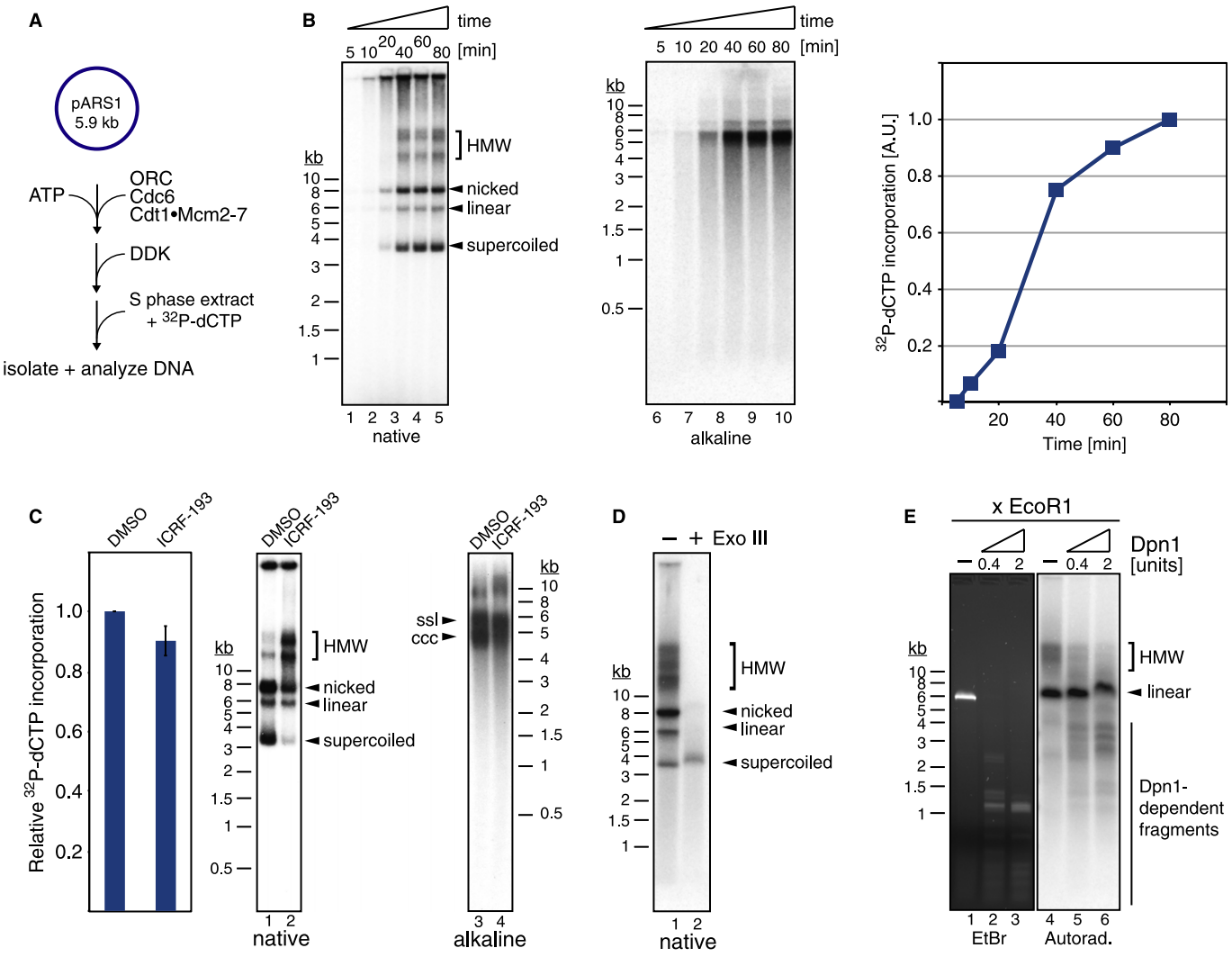

Figure 2. ***In vitro*** **replication of free plasmid DNA.**

A Reaction scheme.

B Time-course analysis of pARS1 replication. Replication products were analyzed by autoradiography after native (lanes 1–5) or denaturing alkaline (lanes 6–10) agarose gel electrophoresis. Total ^{32}P-dCTP incorporation as determined by phosphorimaging is plotted over time on the right.

C Replication in S-phase extract containing 1.25% DMSO (lanes 1, 3) or 177 μM ICRF-193 (lanes 2, 4). Replication products were analyzed by autoradiography after native (lanes 1, 2) or alkaline (lanes 3, 4) agarose gel electrophoresis. Histogram on the left shows total ^{32}P-dCTP incorporation as determined by PhosphorImager analysis; averages and standard deviations of three experimental replicates are shown. HMW: high molecular weight DNA; ssl: single-stranded linear; ccc: covalently closed circular.

D Replication reactions were carried out as in (A), DNA isolated from the replication reaction, and either mock-treated (lane 1) or treated with exonuclease III (Exo III, lane 2) prior to native agarose gel electrophoresis.

E DNA isolated from a replication reaction was either mock-treated (lanes 1, 4), or treated with 0.4 units (lanes 2, 5) or 2 units (lanes 3, 6) of DpnI, and analyzed by native agarose gel electrophoresis. An ethidium bromide stain of the gel is shown on the left (lanes 1–3), the corresponding autoradiogram is depicted on the right (lanes 4–6).

gel. The identity of the negatively supercoiled and nicked DNA species was confirmed by agarose gel electrophoresis in the presence of chloroquine (Supplementary Fig S3); as expected, the gel mobility of nicked plasmid monomer was insensitive to the presence of chloroquine in the gel, whereas the gel mobility of the negatively supercoiled monomer was reduced upon chloroquine intercalation.

Replication forks induce DNA supercoils that can be relaxed by type I and type II topoisomerases, but only type II topoisomerases (Topo II) can decatenate daughter DNA molecules (Wang, 2002). We, therefore, tested whether the generation of plasmid monomers in our system was dependent on Top2 present in the S-phase extract by supplementing the extract with the Topo II inhibitor ICRF-193. While total DNA synthesis was largely unaffected by ICRF-193,

analysis of the replication products by native agarose gel electrophoresis revealed a marked increase in high molecular weight species and a concomitant loss of plasmid monomers (Fig 2C). Alkaline agarose gel electrophoresis revealed that replication products obtained in the presence of ICRF-193 were predominantly close to full-length single-stranded nascent strands (ssl, Fig 2C, lane 4), whereas the generation of the faster migrating circular closed duplex DNA (ccc) was reduced. The identities of the slower and faster migrating bands were confirmed by the pronounced sensitivity of the slower migrating band to Exo III treatment (Supplementary Fig S4), and similar products were also observed previously during SV40 DNA replication *in vitro* (Ishimi *et al*, 1992). This suggests that Topo II inhibition by ICRF-193 blocks completion of DNA replication at a late step, as has also been observed in *Xenopus* extracts before (Cuvier *et al*, 2008). A fraction of the HMW molecules was sensitive to treatment with purified human Topo IIα, suggesting that catenated daughter molecules constitute part of the high molecular weight species (Supplementary Fig S5A). Most of the high molecular weight species, however, also appear to contain a free 3′ end, as demonstrated by the sensitivity to exonuclease III (Exo III; Supplementary Fig S5B). This Exo III sensitivity of the high molecular weight products was independent of ICRF-193 treatment (Fig 2D). Together, these observations suggest that the high molecular weight replication products contain a mixture of replication intermediates, such as precatenanes and partially nicked catenated dimers, and that decatenation of fully replicated plasmid daughters is catalyzed by Top2 in the extract. From this follows that our system supports the initiation, elongation, and termination stages of DNA replication.

Replication of plasmid DNA immobilized on beads yields predominantly shorter than full-length nascent strands (Fig 1). In striking contrast, the majority of replication products obtained with free plasmid DNA was full-length or close to full-length (Fig 2B, lanes 6–10). To exclude the possibility that full-length ^{32}P-labeled DNA strands were the result of gap-filling synthesis, we probed the newly synthesized DNA with DpnI. This enzyme efficiently cleaves the DNA sequence GATC when fully methylated (GA^{m}TC), but exhibits reduced activity toward hemimethylated DNA, and does not cleave unmethylated GATC sequences. Plasmid pARS1 contains 23 copies of the GATC target sequence. To increase the sensitivity of the assay, we linearized the DNA with EcoRI, which collapses the distribution of topoisomers that typically form upon incubation of the plasmids in the extract into a single band of linears. Total DNA was observed by ethidium bromide staining, whereas newly synthesized DNA in the same sample was detected by autoradiography. As shown in Fig 2E, the majority of the fully methylated input plasmid DNA (isolated from a *dam*$^{+}$ *E. coli* strain) was digested at the lowest concentration of DpnI (lanes 1–3), whereas replicated plasmid molecules were resistant to digestion under these conditions (lanes 4–6). Replicated DNA, however, is resistant to cleavage by MboI (Supplementary Fig S6), which recognizes the same GATC sequence as DpnI, but only when this DNA sequence is unmethylated on both strands. Together, these results indicate that replicated DNA molecules are hemimethylated, which is consistent with free plasmid DNA undergoing a single round of replication *in vitro*. The continuous strand synthesis suggested by these observations is corroborated by the fact that ^{32}P-dCTP is incorporated along the length of the plasmid DNA (Supplementary Fig S7). The data in Fig 2E demonstrate that only a small fraction of the input DNA molecules is replicated in our system. As the number of pre-RCs formed on DNA in solution is not known, we are unable to determine pre-RC activation efficiency. However, elongation seems to be efficient once initiation has occurred, as replicated DNA strands are mostly full-length. We, therefore, presume that a step preceding elongation is limiting overall levels of replication.

Plasmid replication in solution exhibits hallmarks of cellular DNA replication initiation

In the approach in Fig 2, pre-RC components are mixed with S-phase extracts, raising the possibility that purified and endogenous pre-RC components interacted in the S-phase extract to form functional pre-RCs. In addition, we noted that circular closed plasmid DNA molecules underwent DNA replication-independent nicking in the extract (Supplementary Fig S8), which could theoretically provide an entry site for a DNA polymerase to perform strand displacement DNA synthesis. To exclude any potential origin-independent mechanisms of replication initiation in the S-phase extract, we re-evaluated the contribution of replication initiation factors to DNA synthesis in our modified system.

DNA synthesis in the soluble system was dependent on purified Cdc6 (Fig 3A, B), ORC (Fig 3B), and Cdt1·Mcm2-7 (Fig 3C), indicating that purified pre-RC components do not form functional pre-RCs with endogenous pre-RC components that may be present in the S-phase extract. This may be expected as all budding yeast pre-RC components are subject to CDK-dependent inhibition in S phase (Diffley, 2011). DNA replication was moreover dependent on DDK and CDK (Fig 3B), and on Mcm10, depletion of which could be rescued by re-addition of purified recombinant Mcm10 to the extract (Fig 3D). Finally, nucleotide incorporation exhibited a pronounced sensitivity to aphidicolin (Fig 3E), confirming a role for replicative DNA polymerases during plasmid replication *in vitro*. In *Xenopus* egg extracts, aphidicolin induces the uncoupling of the CMG helicase from the replicative DNA polymerases, leading to extensive DNA unwinding in the absence of DNA synthesis, which promotes the recruitment of RPA to the excess of single-stranded DNA (Walter & Newport, 2000; Byun *et al*, 2005; Pacek *et al*, 2006). Interestingly, here, we observed that yeast RPA recruitment was inhibited by aphidicolin (Fig 1C, lanes 4 and 5), suggesting that helicase uncoupling from the polymerase does not occur in the yeast system.

ARS-independent budding yeast DNA replication *in vitro*

In bacteria, negative supercoiling of the DNA template is essential for DNA duplex opening by the initiator, DnaA (Bramhill & Kornberg, 1988). Here, we found that negatively supercoiled, relaxed, or linear plasmid forms were replicated with comparable efficiencies, indicating that negative supercoiling is not essential for functional pre-RC formation and replication initiation *in vitro* (Supplementary Fig S9), as has also been observed in the extract-based system previously (Heller *et al*, 2011).

To characterize the DNA sequence requirements for DNA replication *in vitro,* we initially focused our attention on the well-characterized ARS1 origin, which comprises an A element, harboring the essential ACS, and three non-essential B elements (B1-3) (Marahrens & Stillman, 1992). The ACS and B1 elements constitute

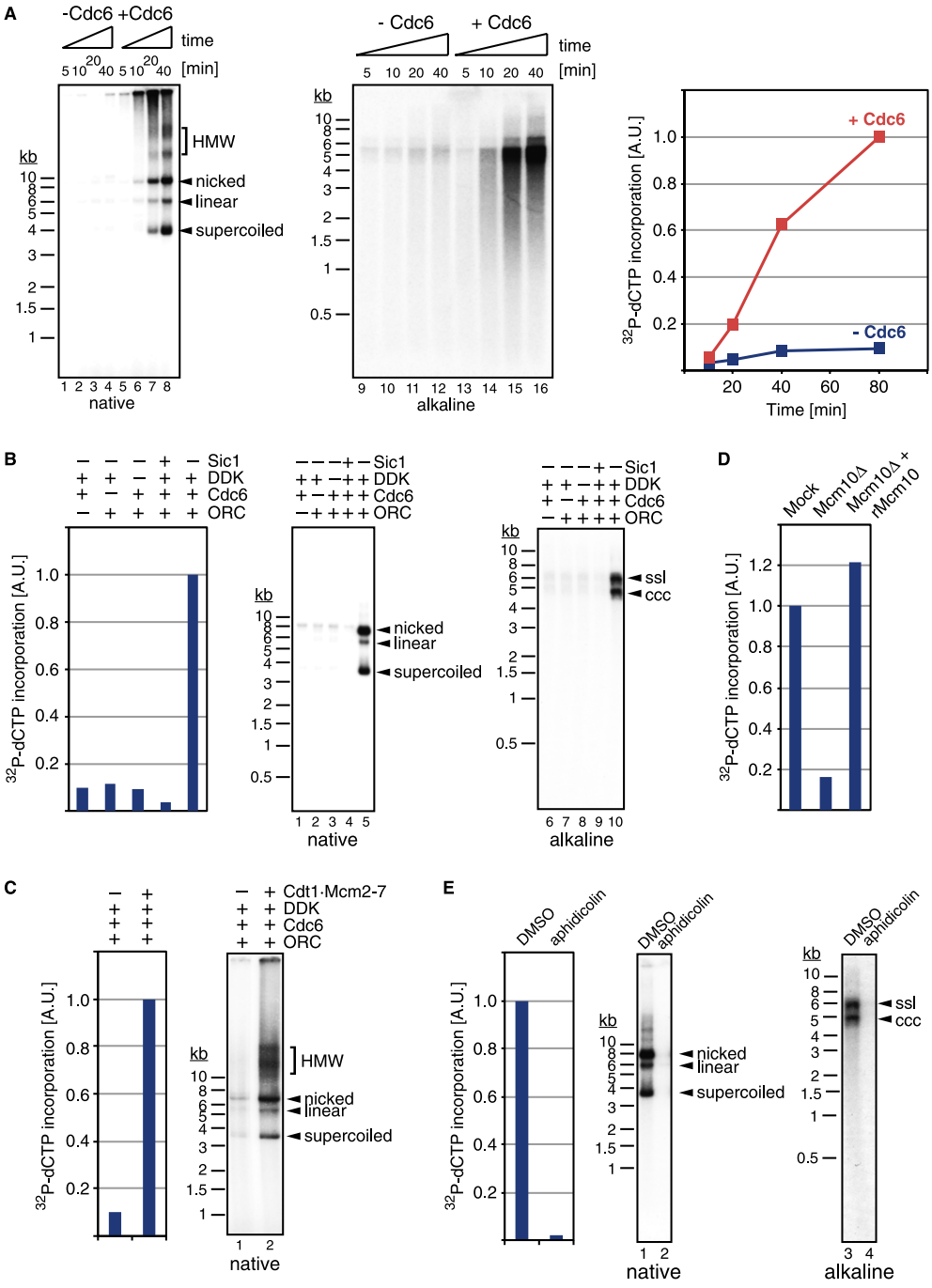

Figure 3. Protein requirements for plasmid replication *in vitro*.

A Time-course analysis of pARS1 replication in the absence (lanes 1–4, and 9–12) or presence (lanes 5–8, and 13–16) of Cdc6 during the pre-RC assembly reaction. Autoradiograms of native (lanes 1–8) or alkaline (lanes 9–16) agarose gel analyses of replication products are shown; total ^{32}P-dCTP incorporation is plotted on the right.

B Replication reactions were carried out in the absence or presence of factors indicated on top of each panel. Replication products were analyzed by native (lanes 1–5) or alkaline (lanes 6–10) agarose gel electrophoresis and autoradiography. Total ^{32}P-dCTP incorporation is plotted on the left.

C *In vitro* replication reaction in which purified Cdt1·Mcm2-7 was omitted (lane 1) or included (lane 2) during pre-RC assembly. Replication products were analyzed by native agarose gel electrophoresis. Histogram depicts the total ^{32}P-dCTP incorporation.

D ^{32}P-dCTP incorporation was monitored in mock-depleted, Mcm10-myc-depleted, or Mcm10-myc-depleted extract supplemented with purified recombinant Mcm10.

E *In vitro* replication was performed in the presence of 1.25% DMSO (lanes 1, 3) or 37 μM aphidicolin (lanes 2, 4). Products were analyzed by native (lanes 1–2) or alkaline (lanes 3–4) agarose gel electrophoresis and autoradiography. Total ^{32}P-dCTP incorporation is plotted on the left.

the ORC binding site (Bell & Stillman, 1992; Diffley & Cocker, 1992; Rao & Stillman, 1995; Rowley *et al*, 1995), the B2 element forms part of the pre-RC assembly site (Diffley *et al*, 1994; Wilmes & Bell, 2002), and the B3 element contains a binding site for the Abf1 transcription factor (Diffley & Stillman, 1988). We compared the *in vitro* replication efficiency of pARS1 containing wild-type, A^- mutant, or an A^-B2^- double-mutant ARS1 (Fig 4). Assays were performed over a range of limiting ORC concentrations, corresponding to molar ratios of ORC to plasmid of 0.5:1 (lanes 2, 6, 10), 1.4:1 (lanes 3, 7, 11), and 4:1 (lanes 4, 8, 12). Unexpectedly, all pARS1 constructs replicated with similar efficiencies over the range of ORC concentrations tested (Fig 4A, B), thus strongly contrasting the strict ARS1 dependence observed for pARS1 replication *in vivo* (Marahrens & Stillman, 1992). This suggests that specific origin sequences, although essential *in vivo*, are not essential for origin function *in vitro*, which correlates with our previous observation that pre-RC assembly *in vitro* is also not dependent on wild-type origin sequences (Remus *et al*, 2009).

Identification of multiple pre-RC assembly sites in pARS1

We considered three possible models to explain the apparent ARS independence for pARS1 replication *in vitro*: (i) pre-RCs may assemble randomly throughout pARS1; (ii) pre-RCs may assemble with similar efficiencies at ARS1 and a limited number of additional defined sites within pARS1; (iii) ARS1 is the preferred pre-RC assembly site within pARS1, but secondary sites can substitute for ARS1 when ARS1 is inactivated by mutation. The third model is motivated by the observation that ORC binds to an ACS in the B2 element of ARS1 only when the preferred ACS in the A element is disrupted by mutation (Bell & Stillman, 1992).

To identify potential pre-RC assembly sites in pARS1, we fragmented pARS1 into four restriction fragments (Fig 5A) and assessed the ability of the restriction fragments to bind to purified ORC or to load Mcm2-7 using co-immunoprecipitation with Orc1 or Mcm3, respectively. Purified ORC was incubated with the pARS1 restriction fragment mixture, recovered from the binding reaction via a CBP-tag on Orc1, and associated DNA fragments analyzed by agarose gel electrophoresis (Fig 5B). At low concentrations, ORC bound preferentially and with similar affinity to the ARS1-containing 0.9-kb fragment and the CEN4-containing 1.1-kb fragment (lanes 12–14). At higher concentration, ORC associated equally with all DNA fragments. The specific binding of ORC to the ARS1-containing fragment was dependent on ATP (compare lanes 17–19 to lanes 12–14) and the essential ACS of ARS1 (compare lanes 32–34 to lanes 12–14), indicating that ORC bound specifically to ARS1. ORC binding to the CEN4-containing 1.1-kb fragment was stimulated in the presence of ATP (compare lanes 17–19 to 12–14).

To map Mcm2-7 loading sites in pARS1, we assembled pre-RCs on the pARS1 restriction fragment mixture, immunoprecipitated Mcm2-7 complexes from the reaction via a FLAG-tag on Mcm3, and analyzed associated DNA fragments by agarose gel electrophoresis. To differentiate between "recruited" and "loaded" Mcm2-7 complexes, reactions were carried out in the presence of ATPγS or ATP, respectively, and immunoprecipitates were selectively subjected to high-salt washes (Evrin *et al*, 2009; Remus *et al*, 2009). As shown in Fig 5C, Mcm2-7 were preferentially loaded around the CEN4- and ARS1-containing DNA fragments, and Mcm2-7 loading on the ARS1-containing fragment was dependent on ARS1. Despite their larger sizes, the 2.7-kb and 1.2-kb fragments exhibited only limited Mcm2-7 loading activity. Thus, sites of Mcm2-7 loading mirrored sites of ORC binding. These data indicate that pARS1 contains preferential pre-RC assembly sites at both ARS1 and within a region contained in the 1.1-kb CEN4-containing fragment, which may explain the apparent lack of ARS1 dependence for pARS1 replication *in vitro*.

CEN4 contributes to efficient replication initiation at pARS1 *in vitro*

Intrigued by the efficient pre-RC assembly on the CEN4-containing fragment of pARS1, we used DNase1 footprinting to map binding sites for purified ORC within this fragment. We identified a unique ATP-dependent ORC binding site in CEN4 that overlaps the conserved CDE III element and partially extends into CDE II (Fig 6A, B) (Fitzgerald-Hayes *et al*, 1982). The ORC footprint at CEN4 resembles the footprint of purified ORC at ARS1 (Bell & Stillman, 1992) both in

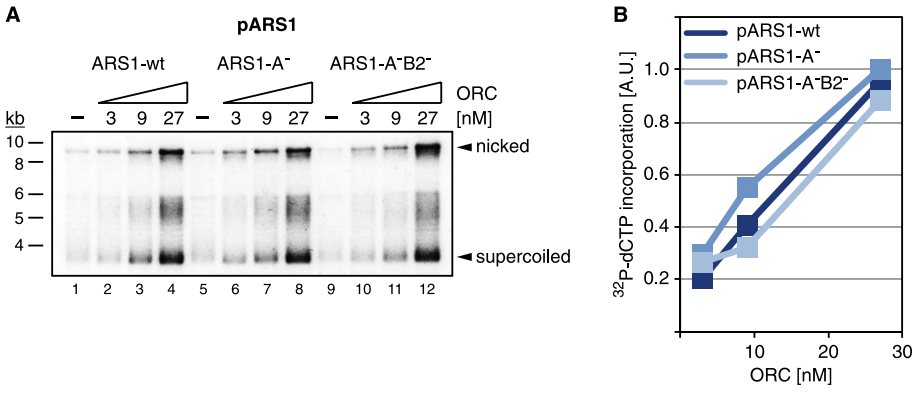

Figure 4. Autonomously replicating sequence (ARS)-independent replication of pARS1 *in vitro*.

A *In vitro* replication of wild-type or mutant pARS1 (7 nM) at various origin recognition complex (ORC) concentrations as indicated. Shown is the autoradiogram of replication products separated by native agarose gel electrophoresis.

B Total ^{32}P-dCTP incorporation, respectively, as in (A).

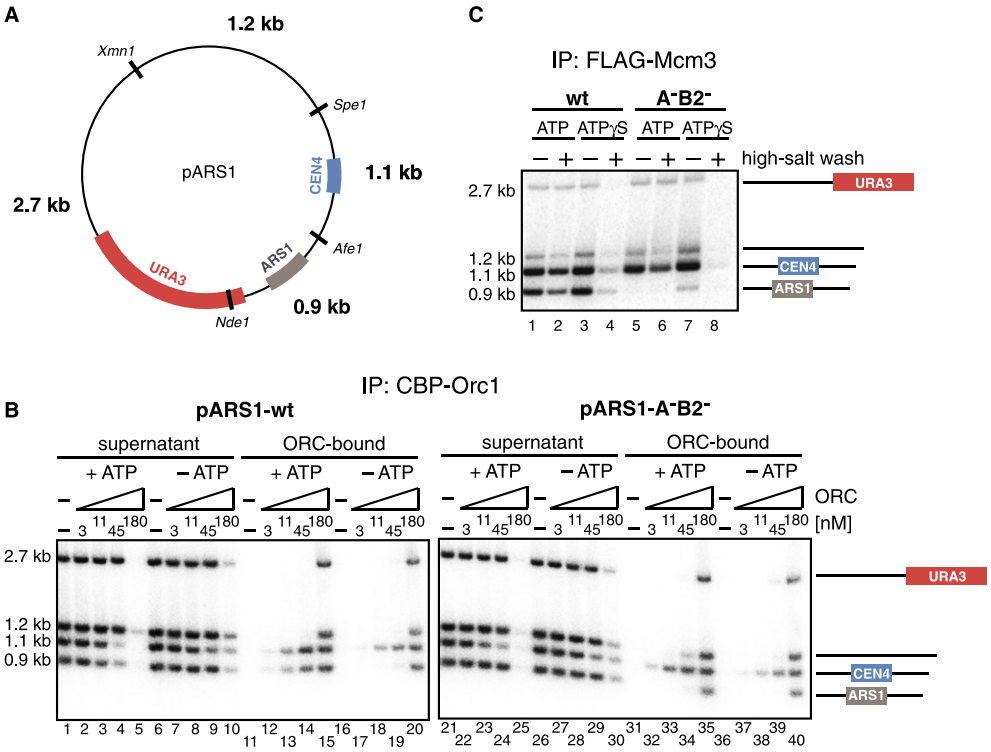

Figure 5. Identification of origin recognition complex (ORC) binding and Mcm2-7 loading sites in pARS1 *in vitro*.

A Map of pARS1 showing the location of restriction sites used for plasmid fragmentation; resulting restriction fragment sizes are indicated in bold font.

B Association of purified ORC with restriction fragments derived from pARS1-wt (lanes 1–20) or pARS1-A$^-$B2$^-$ (lanes 21–40). ^{32}P-labeled fragments co-precipitating with ORC were analyzed by native agarose gel electrophoresis and autoradiography. ORC concentrations are indicated on top. Binding was performed in the presence or absence of ATP as indicated. Fragment cartoons are shown on the right.

C Mcm2-7 loading on pARS1-wt and pARS1-A$^-$B2$^-$ fragments. Fragments co-precipitating with FLAG-Mcm3 in the presence of ATP (lanes 1–2 and 5–6) or ATPγS (lanes 3–4 and 7–8) were analyzed by native agarose gel electrophoresis and autoradiography. Immunoprecipitates were washed with 0.5 M NaCl-containing buffer (high-salt wash) instead of 0.3 M K-glutamate-containing buffer as indicated.

size (~46 bp) and in the generation of asymmetrically positioned DNase1 hypersensitive sites within the region of protection. Sequence analysis of the ORC binding site in CEN4 identifies a 10/11 match to the ACS within CDE III, suggesting that the ORC binding mode at CEN4 is similar to that at ARS elements. However, significant sequence homology between the two ORC binding sites in CEN4 and ARS1 does not extend beyond the ACS (Fig 6B), which is consistent with the lack of extensive sequence conservation between B1 elements (Breier *et al*, 2004; Xu *et al*, 2006; Eaton *et al*, 2010), and with B1 not being essential for specific ORC binding to ARS1 *in vitro* (Bell & Stillman, 1992), despite contributing to ORC binding to ARS1 (Rao & Stillman, 1995; Rowley *et al*, 1995; Lee & Bell, 1997).

The above results suggested that a region overlapping CEN4 acts redundantly with ARS1 *in vitro*. To assess potential origin activity at CEN4 independently from ARS1, we tested the replication efficiency of plasmids bearing mutations at the ACS of CDE III in the ARS1-mutant plasmid pARS1-A$^-$B2$^-$. As shown in Fig 6C, mutation of the ACS at CDE III reduced the replication efficiency of pARS1-A$^-$B2$^-$ by approximately 20% over a range of ORC concentrations, suggesting that CEN4 can promote replication initiation on pARS1 *in vitro*. The modest replication defect indicates that pARS1 contains yet other potential initiation sites. Indeed, we found that mutation of the ACS at CDE III, while eliminating the ATP-dependent ORC footprint at that site (not shown), does not eliminate ORC binding (Supplementary Fig S10) and only slightly reduces Mcm2-7 loading (Fig 6D) on the 1.1-kb CEN4-containing restriction fragment. However, in the absence of the ACS at CDE III, we observed increased levels of Mcm2-7 loading on the 1.2-kb and 2.7-kb restriction fragments, indicating that Mcm2-7 loading is re-distributed to secondary sites in the absence of the ACSs at both CDE III and ARS1 (Fig 6D, compare lanes 6 and 2). It is worth noting that pARS1 contains multiple additional matches to the ACS, which may thus direct replication initiation in the ARS1/CEN4 double-mutant background. Importantly, however, in support of the notion that the 1.1-kb CEN4-containing fragment contains potential origin sites, we show below that this fragment exhibits replicator activity *in vitro* (Fig 9G).

ARS-dependent replication of plasmid DNA *in vitro*

In contrast to pARS1, we found that another ARS-containing plasmid, pARS305, which contains a 7-kb yeast genomic fragment encompassing ARS305, replicated significantly more efficiently in

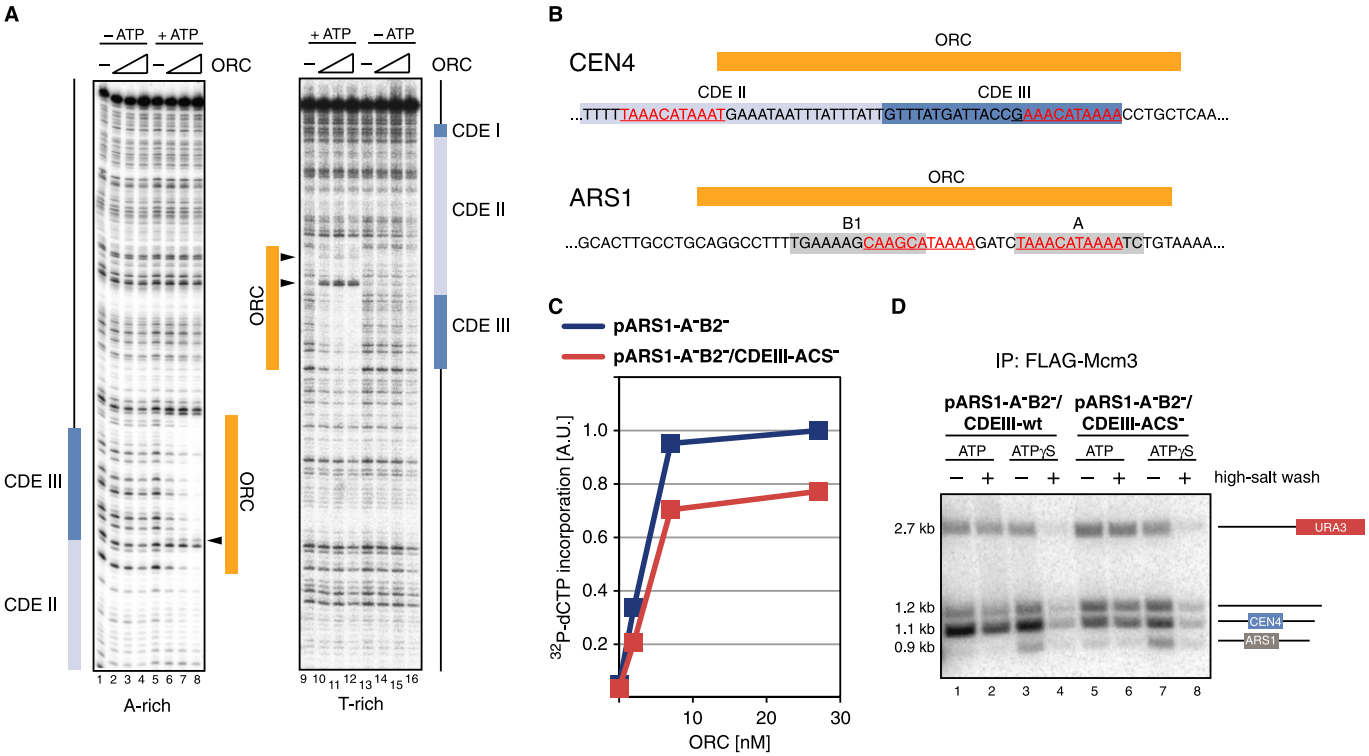

Figure 6. An autonomously replicating sequence (ARS) consensus sequence (ACS) in CEN4 forms part of a pre-RC assembly site *in vitro*.

A DNase1 footprint analysis of purified origin recognition complex (ORC) bound to a CEN4-containing fragment. A-rich denotes the A-rich strand, T-rich denotes the T-rich strand of the ACS in CDE III. Orange boxes indicate the region of DNase1 protection by ORC, arrowheads indicate ORC-induced DNase1 hypersensitive sites. The positions of CEN4 sequence elements are indicated by blue boxes.

B Sequence comparison of the ORC binding sites at CEN4 and ARS1. Orange boxes indicate regions of ORC protection from DNase1 cleavage; ACS matches are highlighted in red underlined font; blue boxes indicate CEN4 elements; gray boxes indicate the positions of the ARS1 A and B1 elements.

C Total ^{32}P-dCTP incorporation as measured by phosphorimaging after *in vitro* replication of pARS1-A$^-$B2$^-$/CDE III-ACS$^-$ or pARS1-A$^-$B2$^-$ in the presence of 0, 2, 7, and 27 nM ORC. Shown are the averages of a duplicate experiment. Error bars are too small to be depicted.

D Mcm2-7 loading on pARS1-A$^-$B2$^-$/CDE III-A$^-$ or pARS1-A$^-$B2$^-$ restriction fragments as measured by co-immunoprecipitation of radiolabelled restriction fragments with FLAG-MCM3.

the presence of wild-type ARS305, compared to an ACS mutant (Fig 7A). Intriguingly, Mcm2-7 loading efficiencies on pARS305-wt and pARS305-A$^-$ were indistinguishable even at low (10 nM) ORC concentrations (Fig 7B), indicating that Mcm2-7 loading alone is not sufficient to license DNA for replication. We characterized ORC binding and Mcm2-7-loading sites on pARS305 restriction fragments as before for pARS1, dividing pARS305 into six restriction fragments (Fig 8A). Consistent with ARS305 being the main origin site in pARS305, ORC associated preferentially with the ARS305-containing 2.1-kb fragment at low concentrations (Fig 8B, lanes 12–13). ORC binding to this fragment was dependent on both ATP (compare lanes 17–18 to lanes 12–13) and the ACS of ARS305 (compare lanes 32–33 to lanes 12–13), indicating that ORC bound specifically to ARS305. At elevated ORC concentrations, additional binding of ORC to the 1.9-kb fragment was observed, while promiscuous binding to all DNA fragments occurred at high ORC concentrations.

Similarly, Mcm2-7 loading was most efficient on the 2.1-kb ARS305-containing fragment, whereas lower levels of Mcm2-7 loading were also detected on the 2.7-kb and 1.9-kb fragments (Fig 8C, lanes 1–4). Mutation of ARS305 reduced the Mcm2-7 loading efficiency at the ARS305-containing fragment, but led to an increase in Mcm2-7 loading on the 2.7-kb and 1.9-kb fragments (Fig 8C, compare lanes 6 and 2), resulting in similar amounts of DNA associated with Mcm2-7. Thus, as observed above for pARS1-A$^-$B2$^-$/CDE III-ACS$^-$, mutation of the primary Mcm2-7 loading site induces a redistribution of Mcm2-7 loading to secondary sites. While compensatory Mcm2-7 loading on sites contained in the 2.7-kb and 1.9-kb fragments may account for the lack of reduction in global Mcm2-7 loading on pARS305-A$^-$ relative to pARS305-wt (Fig 7B), the reduced replication efficiency of pARS305-A$^-$ indicates that pre-RCs loaded at these sites are initiation deficient.

Replication of heterologous plasmid DNAs in budding yeast extracts

The data above demonstrated that both pARS1 and pARS305 assemble functional pre-RCs at their respective ARS elements, but additional sites in each plasmid can also support pre-RC assembly. While pre-RCs assembled outside ARS1 in pARS1 appeared to support replication initiation with similar efficiency as those assembled

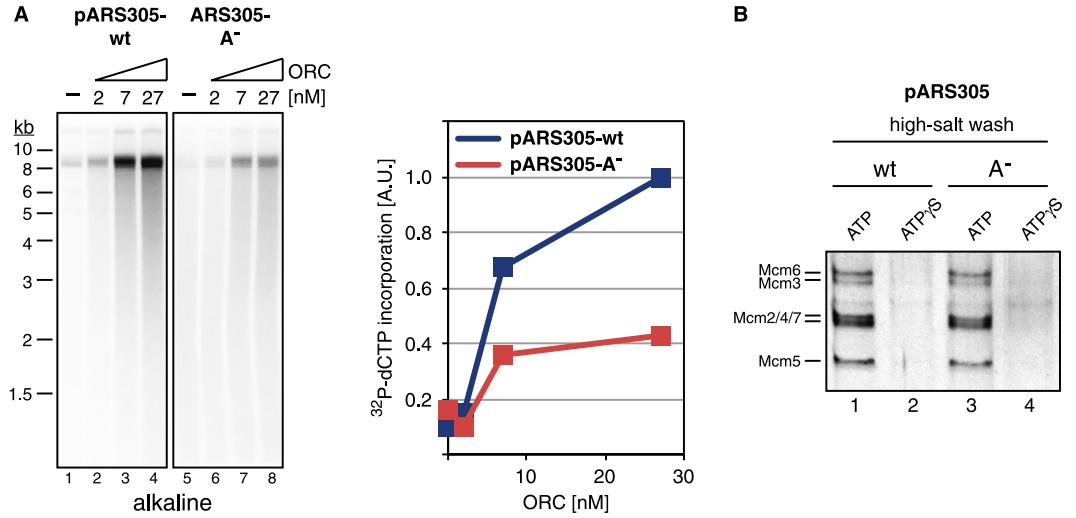

Figure 7. Autonomously replicating sequence (ARS)-dependent replication of pARS305 *in vitro*.

A Replication of pARS305-wt and pARS305-A⁻ (7 nM, respectively) at limiting origin recognition complex (ORC) concentrations *in vitro*. Replication products were analyzed by autoradiography after denaturing alkaline agarose gel electrophoresis (lanes 1–8). Graph shows total ^{32}P-dCTP incorporation, respectively, as determined from duplicate experiments. Error bars are too small to be depicted.

B Reconstituted pre-RCs were assembled using 10 nM ORC in the presence of ATP or ATPγS on bead-coupled wild-type and mutant pARS305, as indicated, washed with 0.5 M NaCl buffer (high-salt wash), and analyzed by SDS–PAGE and silver stain.

at ARS1, pre-RCs assembled outside ARS305 in pARS305 appeared to get activated less efficiently, thus explaining the different ARS dependencies observed for the replication of both plasmids. We, therefore, decided to test entirely heterologous plasmids for their ability to replicate in our cell-free budding yeast replication system (Fig 9). We found that pBluescript supported both efficient Mcm2-7 loading (Fig 9A) and DNA replication *in vitro* (Fig 9B, C). Although Mcm2-7 loading efficiency on the *E. coli* expression vector pET16b was indistinguishable from that on pARS1 even at limiting ORC concentrations (Fig 9D), pET16b replicated with significantly reduced efficiency compared to pARS1 (Fig 9E, F). Thus, pre-RCs license DNA for replication only if loaded around certain DNA sequences. Intriguingly, insertion of a 244 bp ARS1-containing DNA fragment or the 1.1-kb CEN4-containing fragment derived from pARS1 significantly increased the replication efficiency of pET16b (Fig 9G), demonstrating that both ARS1 and the 1.1-kb pARS1-derived non-ARS restriction fragment exhibit yeast replicator activity *in vitro*. These observations demonstrate that although ARS elements are not essential for budding yeast DNA replication *in vitro*, replication initiation *in vitro* is dependent on DNA sequence context.

Discussion

Mcm2-7 double hexamers bound around double-stranded DNA are intermediates of the replication initiation reaction

We have demonstrated that reconstituted budding yeast pre-RCs support cell-free DNA replication. Mcm2-7 double hexamers loaded around double-stranded DNA are, therefore, true intermediates of the eukaryotic DNA replication initiation reaction. Activation of the Mcm2-7 helicase is thus predicted to require the transient opening of the closed Mcm2-7 rings present in the double hexamer and specific extrusion of the lagging strand template in order to form active CMG helicase complexes bound with 3′ to 5′ orientation around the leading-strand template (Moyer *et al*, 2006; Fu *et al*, 2011). This raises the question of how the DNA duplex is opened at eukaryotic origins. In *E. coli*, negative supercoiling of the DNA template is essential for origin unwinding by the initiator, DnaA. In contrast, as demonstrated here and by others previously (Heller *et al*, 2011), negative supercoiling of the template DNA is not required for replication initiation in budding yeast. DNA supercoiling also seems to be dispensable for DNA replication in *Xenopus* extracts (Yardimci *et al*, 2010). Moreover, despite the structural homology of AAA+ subunits of ORC to DnaA (Speck *et al*, 2005; Clarey *et al*, 2006; Erzberger & Berger, 2006), ORC does not exhibit DNA melting activity, which agrees with Mcm2-7 being loaded around double-stranded DNA. Our finding that ORC can be eluted from the DNA after Mcm2-7 loading without disrupting the replication competence of the loaded Mcm2-7 double hexamers suggests that ORC is also not required for duplex opening at a step after Mcm2-7 loading. Initial origin unwinding in eukaryotes, therefore, is likely mediated by a Mcm2-7-dependent mechanism.

The complexity of the Mcm2-7 remodeling reaction during origin activation, which needs to be coordinated with S-phase progression to prevent the generation of unstable single-stranded DNA in the absence of DNA synthesis, may explain the involvement of numerous initiation factors for Mcm2-7 activation. Our demonstration that DDK can be washed away after phosphorylation of purified Mcm2-7 complexes loaded around DNA supports the notion that Mcm2-7 are the only essential targets for DDK during origin activation. We find that DDK and CDK promote the ordered recruitment of the pre-IC

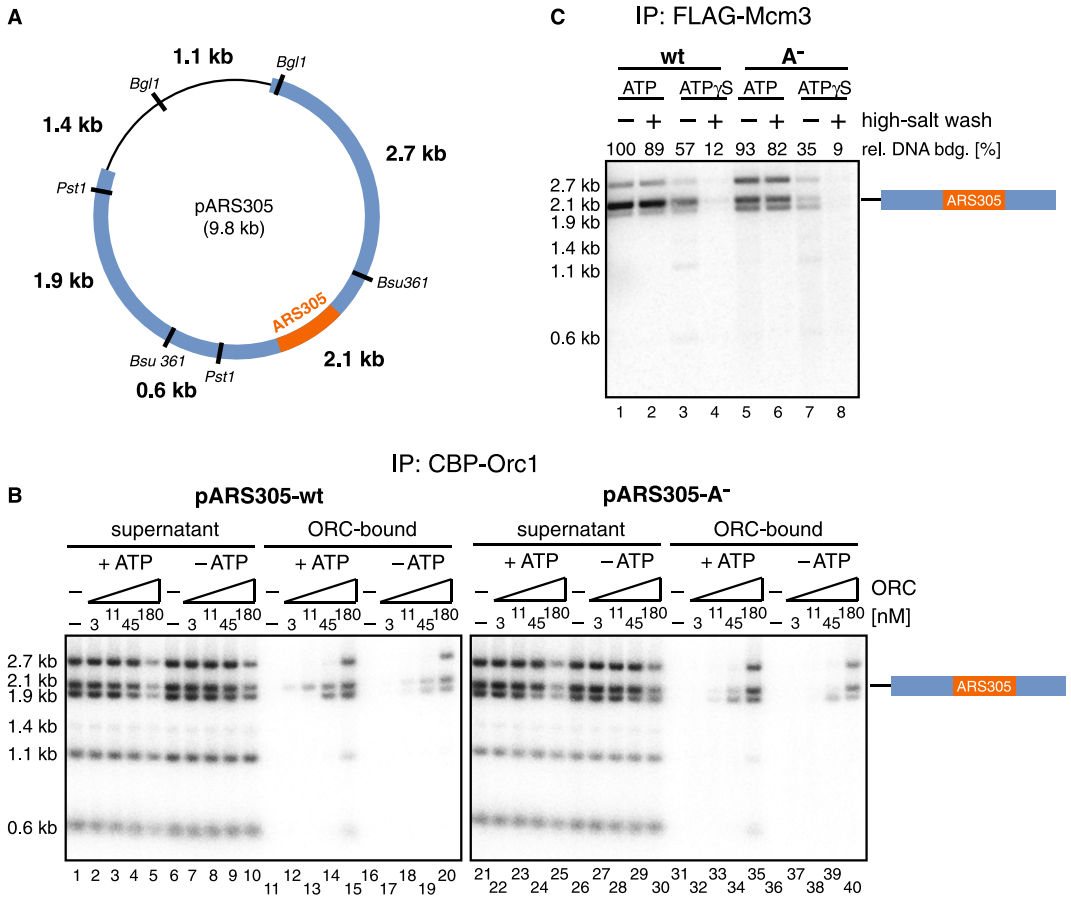

Figure 8. Origin recognition complex (ORC) binding and Mcm2-7 loading sites in pARS305 *in vitro*.

A Map of pARS305. Orange and blue boxes demarcate the genomic fragment integrated into pUC19.

B Association of purified ORC with restriction fragments derived from pARS305-wt (lanes 1–20) or pARS305-A$^-$ (lanes 21–40), analogous to Fig 5B.

C Mcm2-7 loading on pARS305-wt and pARS305-A$^-$ fragments as in Fig 5C. Total DNA recovered by IP, as determined by PhosphorImager analysis, is indicated on top in percent of the amount recovered in lane 1.

components Sld3, Cdc45, Dpb11, and Sld2 to reconstituted pre-RCs in a pattern that mimics pre-IC assembly *in vivo*, indicating that reconstituted pre-RCs initiate DNA replication *in vitro* by a physiological mechanism. Budding yeast Mcm10 appears to play a late role in Mcm2-7 helicase activation after pre-IC formation and CMG assembly (Ricke & Bielinsky, 2004; Heller *et al*, 2011; van Deursen *et al*, 2012; Watase *et al*, 2012). In support of this notion, we find that pre-IC assembly *in vitro* occurs in the absence of Mcm10. Some controversy revolves around the timing of budding yeast Mcm10 association with the pre-RC, with some reports suggesting an association of Mcm10 with pre-RCs as early as G1 phase (Ricke & Bielinsky, 2004; van Deursen *et al*, 2012), and others suggesting a pre-IC-dependent recruitment of Mcm10 to origins (Heller *et al*, 2011; Watase *et al*, 2012). Consistent with the latter studies, we observed that stable Mcm10 association with Mcm2-7 in the extract is dependent on both DDK and CDK. The discrepancies in the observed Mcm10 association timing between multiple studies may suggest that origin recruitment of Mcm10 is influenced by experimental conditions, or that the stability of Mcm10 binding to Mcm2-7 increases upon pre-IC formation.

The role of DNA sequence for specifying budding yeast replication origins

Replication of plasmid DNA in our system results in the generation of covalently closed circular daughter molecules, indicating that the system recapitulates all stages of DNA replication, including initiation, elongation, and termination. The fact that heterologous DNA templates can undergo complete replication in our system supports the observation that DNA replication termination in budding yeast is not dependent on specific *cis*-acting DNA sequences (McGuffee *et al*, 2013). However, our observation that pARS1 was replicated *in vitro* regardless of a functional ARS element was unexpected and contrasted sharply with the requirement for ARS1 for replication of this plasmid *in vivo* (Marahrens & Stillman, 1992). Even entirely heterologous DNA, such as pBluescript, was replicated efficiently in our system, indicating that specific origin sequences may not be essential for budding yeast DNA replication *in vitro*. These observations raise the possibility that the budding yeast genome contains cryptic or inefficient origin sites that may assemble pre-RCs in a stochastic manner below the detection limit of genome-wide origin

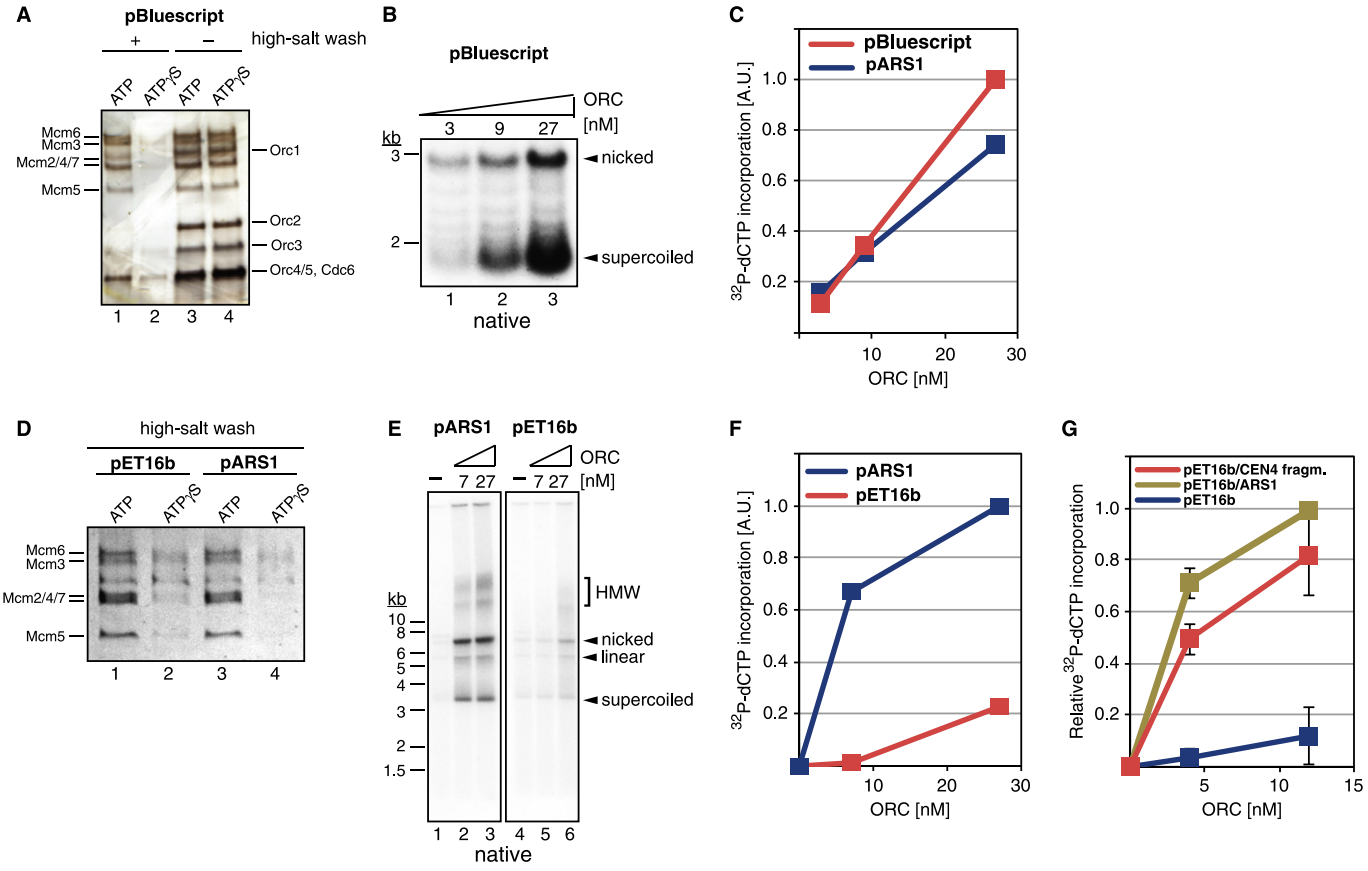

Figure 9. Replication of heterologous DNA templates.

A Reconstituted pre-RC assembly in the presence of ATP or ATPγS on bead-coupled pBluescript, washed with 0.5 M NaCl buffer (high-salt wash) as indicated, and analyzed by SDS–PAGE and silver stain.

B *In vitro* replication of pBluescript. Reactions were performed in the presence of indicated concentrations of origin recognition complex (ORC) and replication products analyzed by autoradiography after native agarose gel electrophoresis.

C Comparison of *in vitro* replication efficiencies of pBluescript and pARS1. Input plasmid concentration was 7 nM. Relative ^{32}P-dCTP incorporation values were normalized for G/C-content and respective plasmid length.

D Reconstituted pre-RC assembly on pET16b and pARS1 (ORC: 10 nM). All reactions were subjected to high-salt wash after assembly and analyzed by SDS–PAGE and silver stain.

E *In vitro* replication of pARS1 and pET16b. Replication products were analyzed by native agarose gel electrophoresis and autoradiography.

F Quantitation of total ^{32}P-dCTP incorporation in experiment in (E).

G Relative *in vitro* replication activity of pET16b, pET16b containing a 254 bp autonomously replicating sequence (ARS) 1 fragment (pET16b/ARS1), and pET16b containing the 1.1-kb CEN4-containing fragment of pARS1 (see Fig 5A; pET16b/CEN4 fragment). Averages and standard deviations of three independent experiments are shown.

mapping techniques, but that may account for chromosomal maintenance in the absence of conserved ARS elements (Dershowitz *et al*, 2007). Such sites may also account for the excess of Mcm2-7 complexes bound to chromosomes in each cell cycle (Blow *et al*, 2011).

Nonetheless, DNA replication in our system does not initiate randomly, as pARS305 depended on ARS305 for efficient replication, while the inefficiency of pET16b replication in our system could be rescued by insertion of ARS1 or a non-ARS/CEN4-containing restriction fragment derived from pARS1. A previous study found that functional revertants of ARS-mutant plasmids most often acquired secondary mutations in the vector backbone that generated new ACS-containing ARSs at these vector sites, rather than reverting the mutant ACS at the original ARS, demonstrating that ACSs in heterologous sequence contexts can function as budding yeast origins *in vivo* (Kipling & Kearsey, 1990). The presence of ACSs in the ectors used here may thus account for the replication competence of heterologous sequences *in vitro*. Consistent with this notion, all plasmids tested here contain multiple matches to the ACS. The conservation of the ACS at budding yeast origins reflects the specific ORC targeting mechanism in this organism, which involves specific and direct binding of ORC to the ACS. However, ORC targeting mechanisms have diversified significantly during the evolution of higher eukaryotes. For example, *Drosophila* and human ORC may bind to DNA with little intrinsic sequence specificity (Vashee *et al*, 2003; Remus *et al*, 2004; Schaarschmidt *et al*, 2004), but may be targeted

to some origins by specific co-factors (Beall *et al*, 2002; Atanasiu *et al*, 2006; Tatsumi *et al*, 2008; Deng *et al*, 2009), while *S. pombe* ORC is targeted to specific DNA structures by a specialized AT-hook DNA binding domain (Chuang & Kelly, 1999). The evolutionarily and developmentally regulated diversification of ORC-origin recruitment mechanisms may thus underlie the apparent lack of conserved origin sequences across eukaryotes.

Mcm2-7 loading is not sufficient for replication origin licensing

Intriguingly, pre-RC assembly sites redistributed around pARS1 *in vitro* upon simultaneous mutation of the predominant sites at ARS1 and CEN4 without greatly affecting replication efficiency of pARS1, indicating that origin usage is flexible *in vitro*. However, this flexibility, or plasticity, in origin usage is limited, as a similar re-distribution of pre-RC assembly sites in pARS305 upon mutation of the essential ACS at ARS305 did not efficiently compensate for the loss of initiation from ARS305. This observation, and the fact that the replication deficient plasmids pET16b and pARS305-A^{-} did not exhibit global defects in Mcm2-7 loading *in vitro*, illustrates that Mcm2-7 loading alone is insufficient to license DNA for replication and that some aspect of DNA sequence contributes to origin function. We have shown here that ORC can be eluted from the DNA after Mcm2-7 loading without disrupting origin activity. While ACSs direct ORC binding, the physical properties of the DNA adjacent to the ACS, rather than a specific DNA sequence, may determine the efficiency of a subsequent step essential for initiation. We speculate that such a step involves the local melting of the DNA duplex by Mcm2-7 complexes or replisome assembly during origin activation. This feature may be shared between budding yeast and higher eukaryotes, and may thus explain the absence of conserved origin DNA sequences outside the ORC binding site in all eukaryotes.

Epigenetic control of replication origin function in budding yeast

Budding yeast origin specification is subject to epigenetic regulation *in vivo*. For example, nucleosomes positioned over ARS1 disrupt its origin activity (Simpson, 1990), which may explain why budding yeast origins are generally located in nucleosome-free intergenic regions (Eaton *et al*, 2010). Chromosomal positioning effects may also explain why not all ARS elements function as origins at their native chromosomal location (Dubey *et al*, 1991; Newlon & Theis, 1993). Our data suggest that a region encompassing CEN4, which does not exhibit origin activity *in vivo*, can support replication initiation *in vitro*. Kinetochores and other chromatin-associated complexes may thus similarly restrict pre-RC formation at certain chromosomal sites. A competition between origin and centromere function may explain why ARS308, which coincides with CEN3, is a highly inefficient origin (Greenfeder & Newlon, 1992). The use of naked DNA templates and reconstituted pre-RCs for origin licensing may bypass restrictions imposed by the chromatin environment and allow pre-RC assembly at sites that would otherwise be sequestered *in vivo*. Similar to higher eukaryotes, epigenetic mechanisms may thus be more important for origin specification in budding yeast than previously appreciated. The system described here may therefore allow for an unbiased assessment of the DNA sequence requirements for eukaryotic DNA replication initiation in the absence of overriding epigenetic mechanisms.

Materials and Methods

Proteins, DNA constructs, and yeast strains

ORC and Cdt1·Mcm2-7 were purified as described (Frigola *et al*, 2013). Cdc6, Sic1, and Mcm10 were expressed in *E. coli*. DDK was purified from yeast cells that conditionally overproduce Cdc7-myc and Dbf4-TAP^{TCP} from the GAL 1,10 promoter. Protein purification protocols, antibodies, plasmids, and yeast strains are detailed in the Supplementary Materials and Methods.

Replication assay

Preparation of S-phase extract and coupling of plasmid DNA to magnetic beads was carried out essentially as described (Heller *et al*, 2011). Unless indicated otherwise, pre-RCs were reconstituted with 50 nM ORC, 50 nM Cdc6, and 250 nM Cdt1·Mcm2-7, for 30 min at 30°C on 5 nM plasmid DNA (either coupled to beads or free) in 40 μl of 25 mM Hepes-KOH pH 7.6, 0.12 M KOAc, 10 mM $Mg(OAc)_2$, 0.02% NP-40, 5% glycerol, 1 mM DTT, and 5 mM ATP. To replicate free plasmid DNA, 50 nM of DDK was subsequently added into the pre-RC assembly reaction and incubation was continued for 20 min, before adding 40 μl of S-phase extract supplemented with 5 mM ATP, 0.1 mM dNTPs (dATP/dTTP/dGTP) each, 0.2 mM rNTPs (UTP/GTP/CTP) each, 10 μCi of [α-^{32}P]-dCTP (3,000 Ci/mmol, Perkin Elmer), 40 mM creatine phosphate (Roche), 0.01 mg/ml creatine kinase (Roche), for 45 min at 30°C. Reactions were stopped by adding 15 mM EDTA, 0.2% SDS, and 20 μg of proteinase K, and incubation for 30 min at 37°C. DNA was isolated by phenol/chloroform extraction and ethanol precipitation, and fractionated on native (1 × TAE) or alkaline (30 mM NaOH, 2 mM EDTA) 0.8% agarose gels. Gels were dried onto Whatman paper and analyzed by phosphor-imaging.

DNA fragment co-precipitation with ORC and Mcm2-7

pARS1 was digested with AfeI, NdeI, SpeI-HF, and XmnI (all DNA modifying enzymes were from NEB); pARS305 was digested with BglI, Bsu36I, and PstI. Restriction fragments were 5′-end-labeled with γ-^{32}P-ATP (Perkin Elmer) and T4 polynucleotide kinase, and purified by phenol/chloroform extraction and ethanol precipitation.

To identify ORC binding sites, restriction fragment mixtures containing 11 nM of each fragment were incubated with ORC as indicated in 40 μl of 25 mM Hepes-KOH pH 7.6/0.12 M KOAc/10 mM $Mg(OAc)_2$/0.02% NP-40/5% glycerol/1 mM DTT/5 mM ATP for 30 min at 30°C. 2 mM $CaCl_2$ and 15 μl of packed Calmodulin affinity resin (Agilent Technologies) were subsequently added and rotated for 45 min at 22°C. The resin was recovered by centrifugation, washed 2× with 0.2 ml of buffer A (25 mM Hepes-KOH pH 7.6/5 mM $Mg(OAc)_2$/0.02% NP-40/5% glycerol/1 mM EDTA/1 mM EGTA/2 mM 2-mercaptoethanol)/0.3 M K-glutamate, and 1× with buffer K (45 mM Hepes-KOH pH 7.6/0.02% NP-40/10% glycerol/5 mM $Mg(OAc)_2$/0.1 M KOAc/2 mM 2-mercaptoethanol). DNA was eluted from the resin with 0.2% SDS/0.1 mg/ml proteinase K at 37°C for 30 min.

To identify Mcm2-7 loading sites, pre-RCs were reconstituted on 11 nM (each) restriction fragments in a 40 μl reaction volume for

30 min at 30°C. FLAG-Mcm3 was immunoprecipitated with 15 μl of packed magnetic M2 agarose beads (Sigma-Aldrich) for 45 min at 22°C. Beads were washed once with 0.4 ml of buffer A/0.3 M K-glutamate (low-salt), once with buffer A/0.5 M NaCl (high-salt) as indicated, and once with buffer K. Co-precipitating DNAs were eluted with SDS and proteinase K as above.

DNase1 footprinting assay

The 273 bp CEN4-containing DNA fragment was amplified from pARS1 using primers DR920 (GCAAAAGGTCACATGC) and DR980 (GACGATAAAACCGGAAGG), one of which was 5′-^{32}P-end-labeled using γ-^{32}P-ATP and T4-polynucleotide kinase. Reactions were carried out in 25 μl of 25 mM Hepes-KOH pH 7.6/100 mM K-glutamate/0.02% NP-40/5 mM $Mg(OAc)_2$/1 mM $CaCl_2$/1 mM DTT and 1 mM ATP as indicated, including 0.4 nM DNA fragment. Binding reactions were incubated for 10 min at 25°C, upon which 0.1 Kunitz units of DNase1 (Worthington) were added for a further 2 min. Reactions were stopped by adding 50 μl of 1% SDS/0.2 M NaCl/20 mM EDTA/0.25 mg/ml yeast tRNA, DNA purified by phenol/chloroform extraction and ethanol precipitation, and DNA fragments fractionated by 6% urea PAGE.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions
JG and SD designed and carried out experiments and analyzed the data. DR designed and carried out experiments, analyzed the data, and wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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